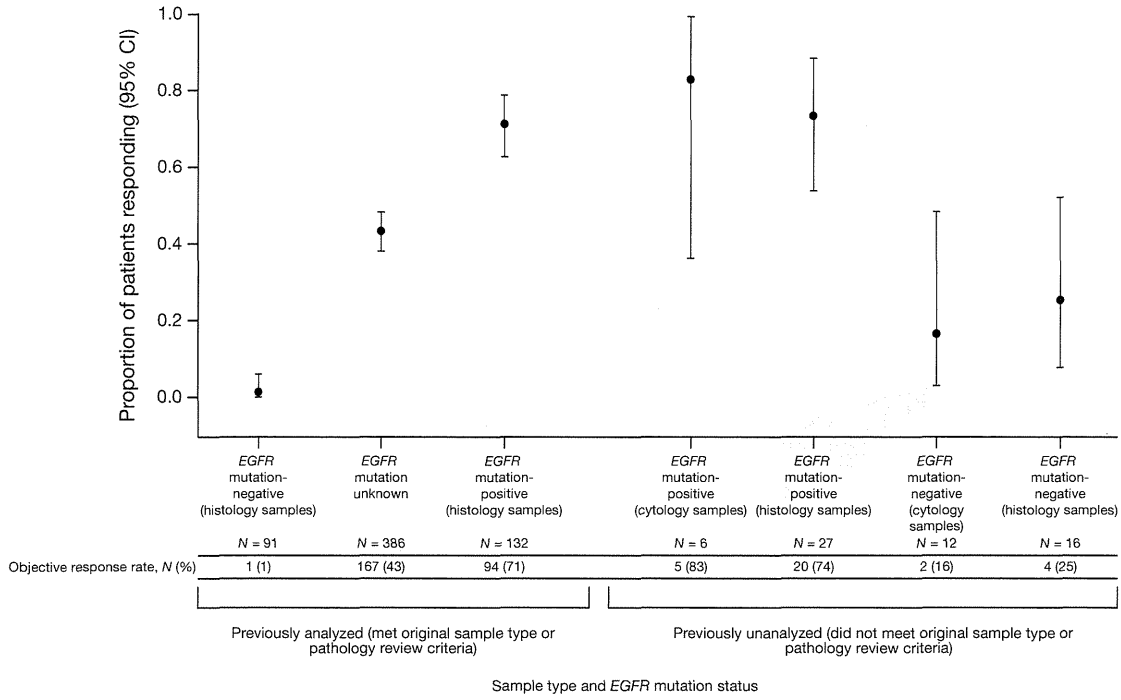


Previously unanalyzed and per-protocol analysis.
Original pathology review criteria included pre-specified thresholds regarding tumor content, sample quality, and sample quantity.
Objective tumor response (complete response or partial response) was determined by the Response Evaluation Criteria in Solid Tumors, version 1.0. Objective response rates were calculated as the percentage of the total number of patients analyzed whose tumors had a confirmed overall response of complete response or partial response.
CI, confidence interval; *EGFR*, epidermal growth factor receptor.

Fig. 4. Objective response rate for patients treated with gefitinib by sample type and *EGFR* mutation status (intent-to-treat population).

patients, including 61 patients described as not previously analyzed and who are described here.

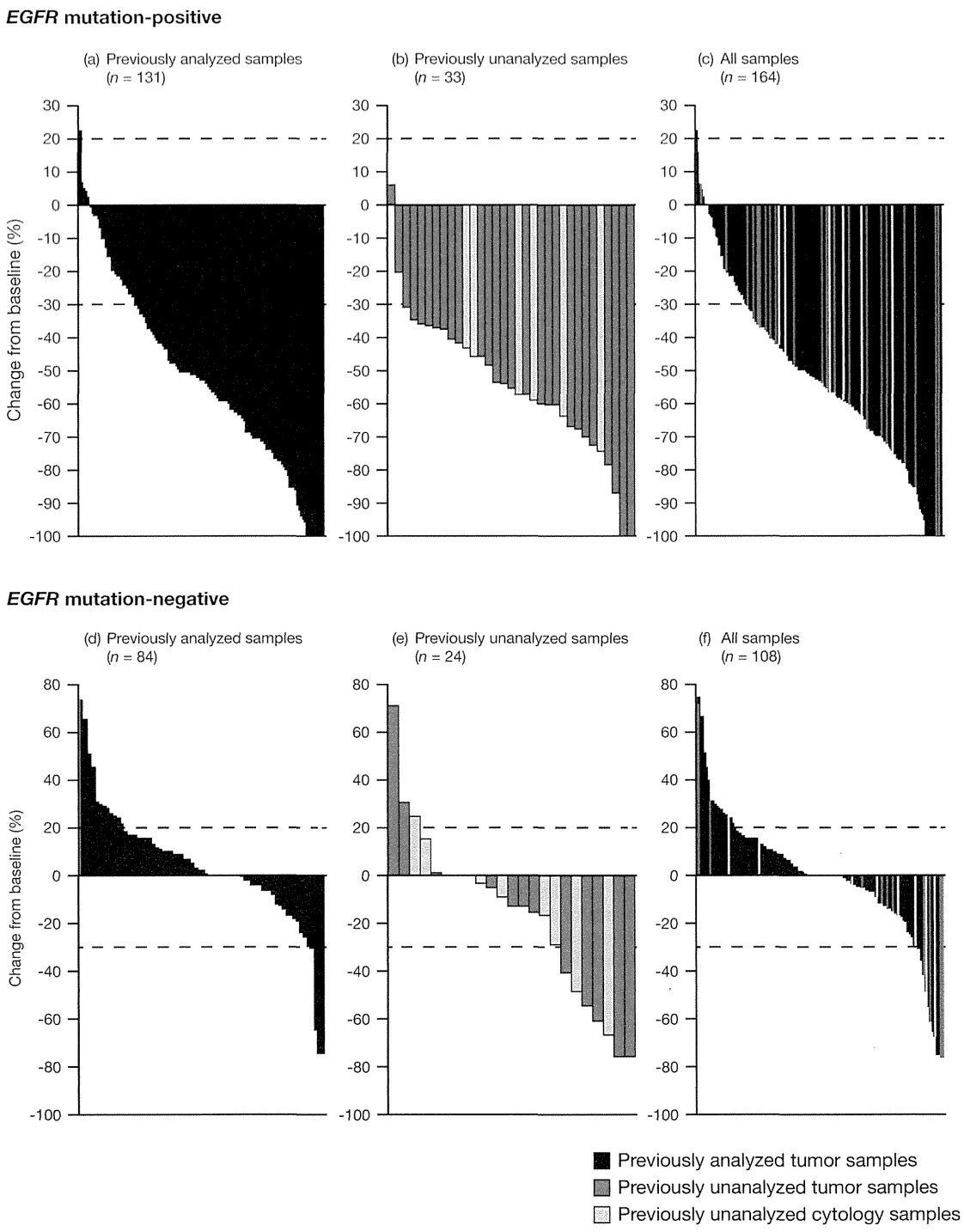
Fig. 4 summarizes the ORR in the previously unanalyzed cytology and histology samples by *EGFR* mutation status for patients with gefitinib. The ORR in the *EGFR* mutation-positive subgroups by cytology and previously unanalyzed histology samples are consistent with the data from the previously determined *EGFR* mutation-positive subgroups: *EGFR* mutation-positive on the basis of cytology ORR 83% ($n = 5/6$), previously unanalyzed histology sample 74% ($n = 20/27$) versus 71% in the previous analysis. The ORR in the *EGFR* mutation-negative subgroups by cytology and previously unanalyzed histology samples are higher than those observed in the previously determined *EGFR* mutation-negative subgroups: *EGFR* mutation-negative on the basis of cytology 16% ($n = 2/12$), previously unanalyzed histology sample 25% ($n = 4/16$) versus 1% in the previous analysis.

Tumor size reduction (percentage change from baseline) with gefitinib in the previously unanalyzed cytology and histology samples appears to be consistent with previously analyzed histology samples, for both *EGFR* mutation-positive (Fig. 5a and b) and -negative samples (Fig. 5d and e). The *EGFR* mutation-positive and -negative tumors from the updated analysis are evenly distributed throughout the waterfall plots of the previously analyzed histology samples (Fig. 5c and f, respectively). Maximum percentage change in tumor size from baseline for patients whose tumors were of unknown *EGFR* mutation status is shown in Fig. 6a (including previously analyzed samples, and cytology and low tumor content samples), Fig. 6b (previously unanalyzed samples highlighting those cytology and low tumor content tumor samples subsequently found to be *EGFR* mutation-positive), and Fig. 6c (previously unanalyzed samples highlighting those cytology and low tumor content tumor samples subsequently found to be *EGFR* mutation-negative).

4. Discussion and conclusions

The results of IPASS clearly demonstrated the differential efficacy of EGFR-TKIs in the *EGFR* mutation-positive, -negative, and -unknown subgroups [4,5]. EGFR-TKIs are now recommended for the treatment of patients with *EGFR* mutation-positive tumors [15]. As a result of available data, accurate identification of patients who might benefit from EGFR-TKI therapy has become an important step in the treatment-decision pathway for advanced NSCLC [16].

This study shows that both histology and cytology samples used to diagnose NSCLC are suitable for the detection of *EGFR* mutations. This study demonstrates that where an *EGFR* mutation-positive result is observed, EGFR-TKI efficacy is consistent with that observed in the sample analysis according to the protocol, albeit with wider ORR CIs due to sample number. In both the cytology and previously unanalyzed histology subgroups, a higher response rate was observed in samples in which no *EGFR* mutation was detected compared with the 1% response rate in the previously analyzed histology samples in which no mutation was detected. While the *EGFR* mutation frequency is as expected in the previously unanalyzed histology samples, it was lower than expected in the cytology samples. Taken together, these two observations demonstrate that there are likely to be a number of false-negative results within the *EGFR* mutation-negative (or *EGFR* mutation-not-detected) subgroups in these previously unanalyzed samples, showing that the *EGFR* mutation-negative results are less robust than in the previously analyzed samples of good quality/quantity. This study therefore demonstrates that while high quality and high tumor content samples should be obtained and tested where possible, it is feasible to use low tumor content or cytology samples if these are the only sample available from the initial diagnosis of advanced NSCLC. Additionally, feedback from pathologists and molecular biologists on sample quality would help to minimize



The horizontal dashed lines at -30% and +20% represent the percentage change required for a response or progression of target lesions, respectively, according to Response Evaluation Criteria In Solid Tumors, version 1.0.
Only patients with a baseline and one evaluable post-baseline target lesion assessment are included. Plots do not include assessment of non-target or new lesions.

EGFR, epidermal growth factor receptor.

Fig. 5. Waterfall plots for maximum percentage change in tumor size from baseline in patients with *EGFR* mutation-positive tumors treated with gefitinib from (a) previously analyzed samples, (b) previously unanalyzed samples, and (c) all analyzed samples; and *EGFR* mutation-negative tumors treated with gefitinib from (d) previously analyzed samples, (e) previously unanalyzed samples, and (f) all analyzed samples.

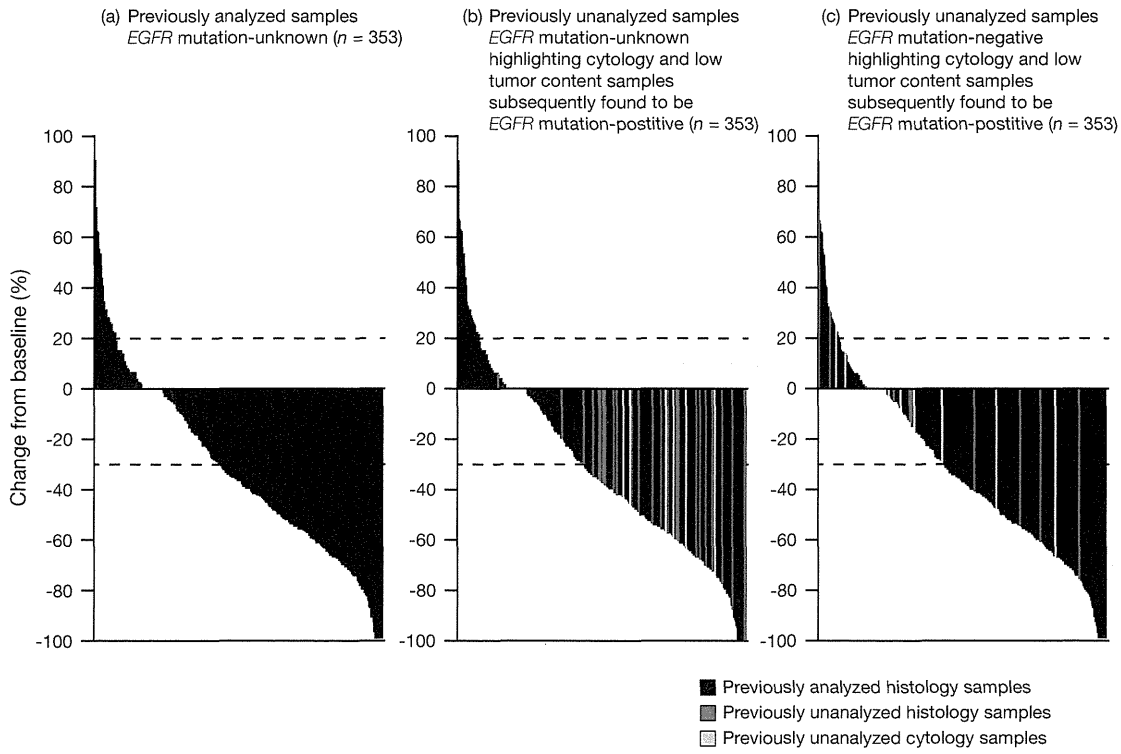


Fig. 6. Waterfall plots for maximum percentage change in tumor size from baseline in patients with tumors of unknown *EGFR* mutation status treated with gefitinib from (a) previously analyzed samples, (b) previously unanalyzed samples with *EGFR* mutation-positives from updated analysis, and (c) previously unanalyzed samples with *EGFR* mutation-negatives from updated analysis.

the costs of repeat testing and optimize the process of obtaining a quality result that the physician can take into consideration when making a treatment decision.

The importance of ensuring that samples are of sufficient quality/quantity has been confirmed in this study. The *EGFR* mutation frequency observed in the cytology samples implies that the prespecified tumor content of 100 cells is still relevant within the clinical setting in order to avoid the issue of false-negative results in this sample type. In contrast, these data suggest that for histology sample analysis, it may be possible to reduce the criteria.

Several groups have released recommendations for *EGFR* mutation testing practices which include guidance on good quality/quantity samples, but little guidance on how laboratories should deal with low tumor content or cytology samples [17–20]. Any samples used for diagnosis of NSCLC (e.g. biopsy, resection, cytology) should be tested for *EGFR* mutation status provided the laboratory performing the analysis is confident in the result. This confidence will depend on the method used, laboratory expertise, and the quality/quantity of the samples, typically those that contain sufficient tumor material to obtain an accurate result, regardless of sample source. Testing of samples judged to be of low quality or low tumor content should be carried out using sensitive testing methods with or without a technique such as Laser Capture Microdissection (LCM), to enrich for the tumor cells. This technique was not attempted in IPASS, because while the technology is available in some institutions, it is not widely available and therefore not possible for all routine *EGFR* testing labs to employ. The Molecular Assays in NSCLC Working Group highlighted that LCM may be used to facilitate accurate test results by increasing the ratio of tumor to normal tissue, which is particularly important for techniques such as direct sequencing, which requires samples with ≥50–70% tumor cells for analysis [17]. However, the Working Group also noted that LCM can be laborious, and is unlikely to be acceptable for routine clinical sample analysis.

This analysis of previously unanalyzed samples from IPASS has shown that NSCLC samples of either low tumor cell content or cytological origin are suitable for the detection of *EGFR* mutation-positive disease. While consideration should be given to the individual capabilities of diagnostic laboratories, the testing of these additional samples may lead to an increase in the number of successful mutation results, enabling a greater number of patients to be accurately diagnosed, and receive the most effective and personalized therapy.

Role of the funding source

This work was supported by AstraZeneca, UK.

Conflict of interest statement

J.C.-H. Yang has received advisory fees from AstraZeneca, Roche, Genentech, Pfizer, and Clovis, and has been an uncompensated advisor to Boehringer Ingelheim and Eli Lilly. Y.-L. Wu and K. Nakagawa have received speaker fees from AstraZeneca.

G. McWalter and R. McCormack are employees of AstraZeneca and hold shares in AstraZeneca. T.S. Mok has received research funding from AstraZeneca and advisory fees from AstraZeneca, Roche, Eli Lilly, Boehringer Ingelheim, Merck Serono, and Pfizer. M. Fukuoka, N. Saijo, V. Chan, and J. Kurnianda have no conflicts of interest to disclose.

Acknowledgements

The authors would like to thank the patients and investigators for their participation in the IPASS study. Sample analysis was performed by Dr Guanshan Zhu, Dr Li Zheng, and Dr Peter Lu at Innovation Center China (China cohort) and Genzyme genetics (non-China samples). Statistical analysis was performed by Dr Rosie Taylor from AstraZeneca, UK. Editing support funded by AstraZeneca was provided by Sarah Lewis, from Complete Medical Communications.

References

[1] Azzoli CG, Baker Jr S, Temin S, Pao W, Aliff T, Brahmer J, et al. American Society of Clinical Oncology Clinical Practice Guideline update on chemotherapy for stage IV non-small-cell lung cancer. J Clin Oncol 2009;27:6251–66.

[2] Azzoli CG, Temin S, Aliff T, Baker Jr S, Brahmer J, Johnson DH, et al. 2011 focused update of 2009 American Society of Clinical Oncology Clinical Practice Guideline update on chemotherapy for stage IV non-small-cell lung cancer. J Clin Oncol 2011;29:3825–31.

[3] Kris MG, Johnson BE, Kwiatkowski DJ, Lafrate AJ, Witsuba II, Aronson SL, et al. Identification of driver mutations in tumor specimens from 1,000 patients with lung adenocarcinoma: the NCI's lung cancer mutation consortium (LCMC). J Clin Oncol 2011;29(Suppl.) [abstract CRA7506].

[4] Fukuoka M, Wu Y-L, Thongprasert S, Sunpaweravong P, Leong S-S, Sriuranpong V, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). J Clin Oncol 2011;29:2866–74.

[5] Mok TS, Wu Y-L, Thongprasert S, Yang C-H, Chu D-T, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361:947–57.

[6] Han JY, Park K, Kim SW, Lee DH, Kim HY, Kim HT, et al. First-SIGNAL: first-line single-agent Iressa versus gemcitabine and cisplatin trial in never-smokers with adenocarcinoma of the lung. J Clin Oncol 2012;30:1122–8.

[7] Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010;362:2380–8.

[8] Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol 2010;11:121–8.

[9] Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012;13:239–46.

[10] Sequist LV, Yang JCH, Yamamoto N, O'Byrne KJ, Hirsh V, Mok TSK, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. J Clin Oncol 2013:3327–34, http://dx.doi.org/10.1200/JCO.2012.44.2806.

[11] Zhou C, Wu Y-L, Chen G, Feng J, Liu X-Q, Wang C, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced *EGFR* mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. Lancet Oncol 2011;12:735–42.

[12] Hlinkova K, Babal P, Berzinec P, Majer I, Mikle-Barathova Z, Piackova B, et al. Evaluation of 2-year experience with EGFR mutation analysis of small diagnostic samples. Diagn Mol Pathol 2013;22:70–5.

[13] Smouse JH, Cibas ES, Janne PA, Joshi VA, Zou KH, Lindeman NI. EGFR mutations are detected comparably in cytologic and surgical pathology specimens of nonsmall cell lung cancer. Cancer 2009;117:67–72.

[14] Hagiwara K, Kobayashi K. Importance of the cytological samples for the epidermal growth factor receptor gene mutation test for non-small cell lung cancer. Cancer Sci 2013;104:291–7.

[15] D'Addario G, Früh M, Reck M, Baumann P, Klepetko W, Felip E. Metastatic non-small-cell lung cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 2010;21(Suppl. 5):v116–9.

[16] Keedy VL, Temin S, Somerfield MR, Beasley MB, Johnson DH, McShane LM, et al. American Society of Clinical Oncology provisional clinical opinion: epidermal growth factor receptor (EGFR) mutation testing for patients with advanced non-small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. J Clin Oncol 2011;29:2121–7.

[17] Eberhard DA, Giaccone G, Johnson BE. Biomarkers of response to epidermal growth factor receptor inhibitors in non-small-cell lung cancer working group: standardization for use in the clinical trial setting. J Clin Oncol 2008;26:983–94.

[18] Marchetti A, Normanno N, AIOM-SIAPEC-IAP, Pinto C, Taddei GL, Adamo V, et al. Recommendations for mutational analysis of EGFR in lung carcinoma. Pathologica 2010;102:119–22.

[19] Pirker R, Herth FJ, Kerr KM, Filipits M, Taron M, Gandara D, et al. Consensus for EGFR mutation testing in non-small cell lung cancer: results from a European workshop. J Thorac Oncol 2010;5:1706–13.

[20] Salto-Tellez M, Tsao MS, Shih JY, Thongprasert S, Lu S, Chang GC, et al. Clinical and testing protocols for the analysis of epidermal growth factor receptor mutations in East Asian patients with non-small cell lung cancer: a combined clinical-molecular pathological approach. J Thorac Oncol 2011;6:1663–9.

OPEN

Survival Outcome Assessed According to Tumor Response and Shrinkage Pattern in Patients with *EGFR* Mutation–Positive Non–Small-Cell Lung Cancer Treated with Gefitinib or Erlotinib

Masayuki Takeda, MD, PhD,† Isamu Okamoto, MD, PhD,*‡ and Kazuhiko Nakagawa, MD, PhD**

Introduction: Somatic mutations in the epidermal growth factor receptor gene (*EGFR*) are associated with a marked therapeutic response to EGFR–tyrosine kinase inhibitors (TKIs) in patients with advanced non–small cell lung cancer (NSCLC). Clinical indicators of the likely survival benefit of EGFR-TKI treatment in NSCLC patients with *EGFR* mutations have not been identified, however. We therefore evaluated progression-free survival (PFS) and overall survival (OS) according to tumor response and tumor shrinkage pattern in such patients.

Methods: Among 145 *EGFR* mutation–positive NSCLC patients treated with EGFR-TKIs, 68 individuals were selected for analysis.

Results: Of the 68 selected patients, 6 achieved a complete response (CR), 42 a partial response (PR), and 14 stable disease (SD). Both PFS and OS were significantly longer in patients who achieved a CR or PR than in those who experienced SD. Multivariate analysis showed that a response (CR or PR) to EGFR-TKIs was significantly associated with both PFS and OS. Among the CR/PR group, the median maximal tumor shrinkage relative to baseline was 56%, and the median time to response (TTR) was 4.2 weeks. The subsets of these patients who experienced rapid tumor regression (TTR of ≤4.2 weeks) or a high degree of tumor shrinkage (≥56%) did not show a more favorable PFS or OS compared with those who experienced slow tumor regression or a low degree of tumor shrinkage.

Conclusion: Response (CR or PR) may represent the optimal surrogate for efficacy among *EGFR* mutation–positive NSCLC patients treated with EGFR-TKIs.

Key Words: Epidermal growth factor receptor, Non–small cell lung cancer, Tyrosine kinase inhibitor, Tumor shrinkage, Response, Mutation, Survival.

(*J Thorac Oncol.* 2014;9: 200–204)

*Department of Medical Oncology, Kinki University Faculty of Medicine, Osaka, Japan; †Department of Medical Oncology, Kishiwada City Hospital, Osaka, Japan; and ‡Center for Clinical and Translational Research, Kyushu University Hospital, Fukuoka, Japan.

Disclosure: The authors declare no conflict of interest.

Address for correspondence: Isamu Okamoto, Center for Clinical and Translational Research, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan. E-mail: okamotoi@kokyu.med.kyushu-u.ac.jp

ISSN: 1556-0864/14/0902-0200

The clinical course of EGFR-TKI–treated patients with *EGFR* mutation–positive NSCLC shows substantial variation. The identification of clinically relevant indicators may provide clinicians with information regarding expected disease progression and prognosis. As far as we are aware, however, no previous studies have evaluated survival according to clinical indicators, such as tumor response and tumor shrinkage pattern, for patients with *EGFR* mutation–positive NSCLC. Several studies have investigated surrogate end points of response for association with progression-free survival (PFS) and overall survival (OS) in NSCLC patients treated with cytotoxic chemotherapy. For individuals with advanced NSCLC treated with platinum-based chemotherapy, those showing a partial response (PR) were thus found to have a better survival than those with stable disease (SD) in one study.[1] In contrast, another study found no significant difference between PR and SD groups with respect to PFS or OS.[2] It therefore remains unclear whether SD benefit for patients treated with platinum-based chemotherapy is the same that as the benefit for those who achieve a complete response (CR) or PR. With regard to treatment with EGFR-TKIs in unselected patients with advanced NSCLC, a previous study found that PFS and OS were significantly longer in the CR/PR group than in the SD group.[3] However, such analysis has not been performed for patients with *EGFR* mutation–positive NSCLC. Although such patients have clinical features associated with a rapid and marked reduction in tumor size in response to EGFR-TKI treatment, the impact of such rapid and pronounced tumor shrinkage on survival outcome has remained unknown. We have therefore now evaluated PFS and OS according to response and tumor shrinkage pattern among *EGFR* mutation–positive NSCLC patients treated with EGFR-TKIs.

PATIENTS AND METHODS

We screened 145 consecutive patients with *EGFR* mutation–positive NSCLC who were treated with EGFR-TKIs between May 2003 and July 2012 at Kinki University Hospital or Kishiwada City Hospital. Criteria for use of a patient's data included the provision of signed informed consent for *EGFR* mutation analysis, a diagnosis of stage IIIb or IV or recurrent NSCLC with a proven *EGFR* mutation, the presence of at least one tumor lesion that could be accurately measured by

computed tomography according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1., and treatment with gefitinib or erlotinib. All patients were evaluated at least every 8 weeks until response confirmation by RECIST. Maximal tumor shrinkage was defined as the greatest tumor shrinkage achieved at any follow-up assessment. Time to response (TTR) was defined as the time from the start of treatment with an EGFR-TKI to the first objective tumor response (tumor shrinkage of ≥30%) observed for patients who achieved a CR or PR. OS and PFS were assessed from the first day of EGFR-TKI therapy to the date of death from any cause and the date of objective disease progression, respectively. The study protocol was approved by the institutional review board at each study site.

RESULTS

Patient Characteristics

Among 145 *EGFR* mutation–positive NSCLC patients treated with EGFR-TKIs, 68 individuals were selected for analysis (Fig. 1). There were no substantial differences in patient characteristics between eligible (n = 68) and ineligible (n = 77) patients (Supplementary Table S1, Supplemental Digital Content 1, http://links.lww.com/JTO/A497). Demographics of the eligible 68 patients are shown in Table 1. Fifty-seven (84%) of these individuals were treated with gefitinib and 11 (16%) with erlotinib. Fifty-two patients (76%) were women and 52 (76%) were never-smokers, with the median age of all patients being 69 years (range, 39–87). Sixty-seven patients (99%) had adenocarcinoma, and 59 (87%) had disease of stage IIIb or IV. Most patients (90%) had a good Eastern Cooperative Oncology Group performance status (0 or 1), and 38 (56%) received EGFR-TKI treatment as first-line chemotherapy. With regard to the type of *EGFR* mutation, 34 patients (50%) had a deletion in exon 19, 31 (46%) had a missense mutation in exon 21 (L858R or L861Q), and three (4%) had a G719A mutation in exon 18.

Analysis of PFS and OS According to Response to EGFR-TKI Treatment

According to the RECIST criteria, six patients experienced a CR, 42 patients a PR, 14 patients SD, and six patients progressive disease (PD) (Table 1). The response rate (CR + PR) and disease control rate (CR + PR +SD) were thus 71% (48 of 68 patients) and 91% (62 of 68 patients), respectively. For the entire cohort, the median PFS was 11.3 months (95% confidence interval [CI], 7.5–15.2) and the median OS was 24.9 months (95% CI, 8.8–40.9). Analysis of PFS according to response to EGFR-TKI treatment revealed a significant benefit for the CR/PR group compared with the SD group (median of 15.9 versus 8.5 months, p = 0.009) (Fig. 2*A*). Kaplan–Meier curves for OS also revealed a significant benefit for the CR/PR group compared with the SD group (median of 44.4 versus 12.2 months, p = 0.004) (Fig. 2*B*).

To rule out potential confounding interaction between response and other factors, we performed multivariate analysis for PFS and OS (Table 2). Response (CR or PR) to EGFR-TKI treatment (hazard ratio [HR], 0.33; 95% CI, 0.17–0.62; p = 0.001) and a favorable performance status (HR, 0.25; 95% CI, 0.10–0.65; p = 0.004) were significantly associated with PFS, whereas response (CR or PR) to EGFR-TKI treatment (HR, 0.29; 95% CI, 0.13–0.68; p = 0.004), a favorable performance status (HR, 0.18; 95% CI, 0.05–0.65; p = 0.008), and female sex (HR, 0.22; 95% CI, 0.06–0.79; p = 0.021) were

TABLE 1. Characteristics of the Enrolled NSCLC Patients with *EGFR* Mutations (n = 68)

Characteristic	Subset	No. of patients (%)
Sex	Male	16 (24)
	Female	52 (76)
Median (range) age in years		69 (39–87)
Smoking history	Never-smoker	52 (76)
	Smoker	16 (24)
Tumor histology	Adenocarcinoma	67 (99)
	Squamous cell carcinoma	1 (1)
ECOG performance status	0–1	61 (90)
	2–3	7 (10)
Disease stage	IIIb	9 (13)
	IV	50 (74)
	Postoperative recurrence	9 (13)
No. of previous chemotherapies	0	38 (56)
	1	22 (32)
	≥2	8 (12)
EGFR mutation	Deletion of exon 19	34 (50)
	L858R mutation in exon 21	30 (44)
	L861Q mutation in exon 21	1 (1)
	G719A mutation in exon 18	3 (4)
EGFR-TKI	Gefitinib	57 (84)
	Erlotinib	11 (16)
Response to EGFR-TKI	Complete response	6 (9)
	Partial response	42 (62)
	Stable disease	14 (21)
	Progressive disease	6 (9)

EGFR-TKI, epidermal growth factor receptor gene tyrosine kinase inhibitor; ECOG, Eastern Cooperative Oncology Group.

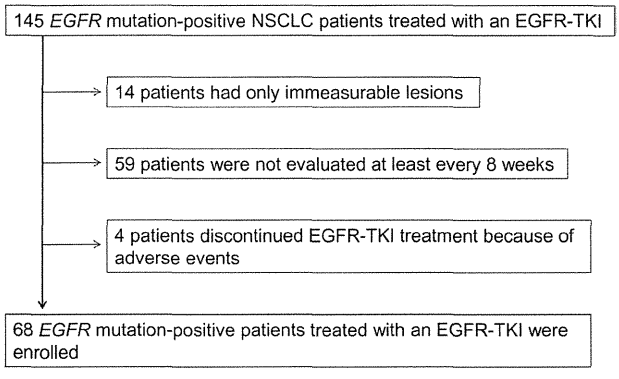


FIGURE 1. Flowchart of patient selection. EGFR-TKI, epidermal growth factor receptor gene tyrosine kinase inhibitor; NSCLC, non–small-cell lung cancer.

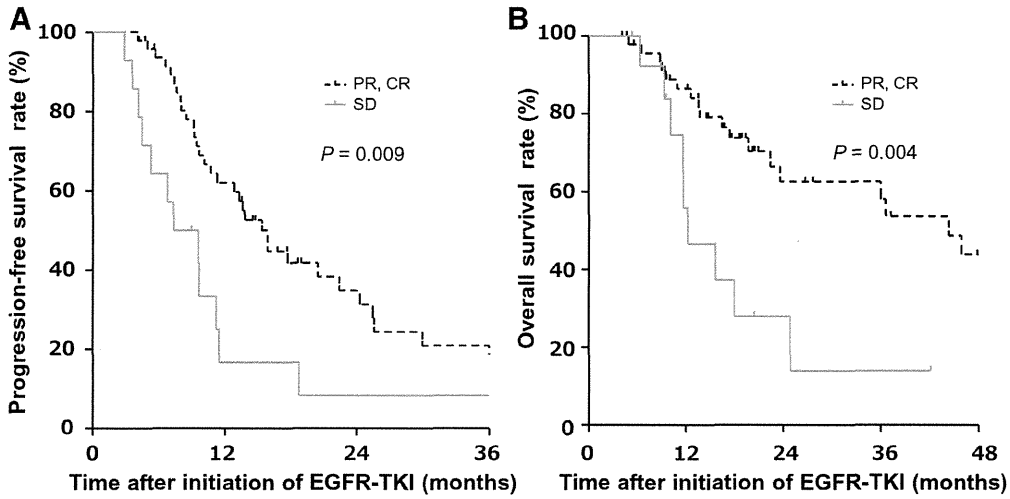


FIGURE 2. Progression-free survival (*A*) and overall survival (*B*) for patients classified according to achievement of a CR or PR response versus stable disease SD. EGFR-TKI, epidermal growth factor receptor gene tyrosine kinase inhibitor; CR, complete response; PR, partial response; SD, stable disease.

significantly associated with OS. Other covariables (smoking history, age, *EGFR* genotype, and type of mutation) did not affect PFS or OS.

Relation of Maximal Tumor Shrinkage to PFS

Given that a response to EGFR-TKI treatment was found to be associated with a longer PFS and OS, we investigated the impact of a marked reduction in tumor size on survival outcome among patients who achieved a CR or PR. The maximal decrease in tumor size over time ranged from 30% to 100% for this group, with a median value of 56% (Fig. 3). No significant correlation was detected between maximal tumor shrinkage and PFS (R^2 = 0.0008), however (Fig. 4*A*). We divided this group of patients into two subgroups according to maximal tumor shrinkage (low shrinkage, <56%; high shrinkage, ≥56%), but no trend toward a more favorable PFS (p = 0.87) (Fig. 4*B*) or OS (p = 0.55) (Fig. 4*C*) was apparent in the subset of patients who experienced a more pronounced change in tumor size in response to EGFR-TKI therapy.

PFS According to TTR

We next investigated the impact of rapid tumor shrinkage, as reflected by TTR, on survival outcome among patients who achieved a CR or PR. The median TTR was 4.2 weeks (95% CI, 3.9–4.5), with most patients (97%) achieving a CR or PR within 2 months after initiation of EGFR-TKI treatment. No correlation was apparent between TTR and PFS (R^2 = 0.0084; Fig. 4*D*). We divided this group of patients into two subgroups according to TTR (long TTR, >4.2 weeks; short TTR, ≤4.2 weeks), but there was no significant difference in PFS (p = 0.29; Fig. 4*E*) or OS (p = 0.58; Fig. 4*F*) between patients with a long or a short TTR.

DISCUSSION

EGFR-TKIs such as erlotinib and gefitinib are highly effective for the treatment of NSCLC patients harboring activating *EGFR* mutations.[4–7] The efficacy of EGFR-TKI treatment varies, however, even among *EGFR* mutation–positive NSCLC patients, with no studies to date having evaluated

TABLE 2. Multivariate Analysis for Survival after Initiation of EGFR-TKI Treatment in NSCLC Patients with *EGFR* Mutations (n = 68)

	Progression-Free survival			Overall Survival		
Factor	**HR**	**95% CI**	p	**HR**	**95% CI**	p
Sex (female/male)	0.37	0.14–1.03	0.056	0.22	0.06–0.79	**0.021**
ECOG PS (0–1/2–3)[a]	0.25	0.10–0.65	**0.004**	0.18	0.05–0.65	**0.008**
Smoking history (never-smoker/smoker)	1.19	0.48–2.96	0.716	2.2	0.74–6.59	0.156
Age (≤ 69/>70 years)	1.00	0.52–1.92	0.999	0.96	0.43–2.14	0.917
EGFR mutation (E19del/other)	0.66	0.35–1.23	0.190	0.49	0.21–1.14	0.096
EGFR-TKI (erlotinib/gefitinib)	0.96	0.38–2.42	0.928	1.59	0.53–4.82	0.409
Response ([CR + PR]/[SD + PD])	0.33	0.17–0.62	**0.001**	0.29	0.13–0.68	**0.004**

[a]At initiation of EGFR-TKI treatment.

EGFR-TKI, epidermal growth factor receptor gene tyrosine kinase inhibitor; ECOG, Eastern Cooperative Oncology Group; CI, confidence interval; HR, hazard ratio; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; PS, performance status; E19del, exon-19 deletion.

p values <0.05 are shown in bold.

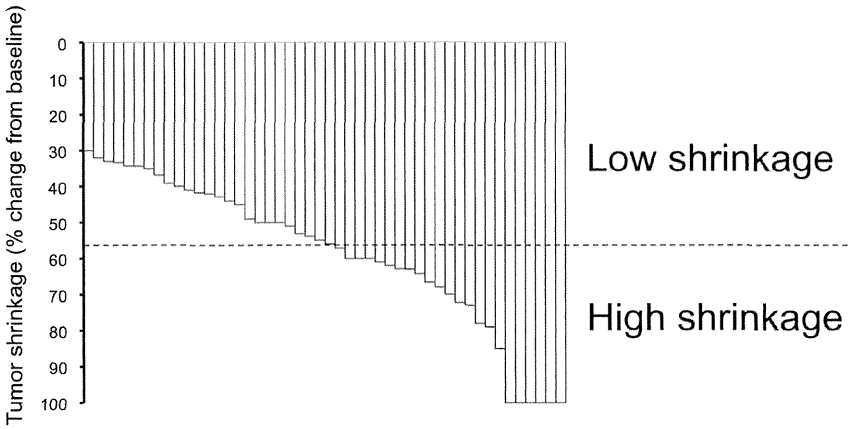


FIGURE 3. Waterfall plot of the maximal decrease in tumor size over time relative to the pretreatment baseline for individual patients treated with epidermal growth factor receptor gene tyrosine kinase inhibitor who achieved a partial response or complete response. The median decrease of 56% was used to define patient subgroups characterized by low or high tumor shrinkage.

survival according to tumor response among such individuals. We have now shown that PFS and OS were significantly longer in such patients who achieved a CR or PR than in those who manifested SD. Multivariate analysis also revealed that a response (CR or PR) to EGFR-TKI treatment was associated with a longer PFS and OS, suggesting that response might represent the optimal surrogate for efficacy in patients with *EGFR* mutation–positive tumors treated with EGFR-TKIs.

Although SD is a relatively more complex category than CR or PR, ranging from a minor decrease to a minor increase in tumor size, we found that most *EGFR* mutation–positive NSCLC patients who experienced SD with EGFR-TKI therapy showed some tumor shrinkage, ranging from 4% to 27% relative to baseline, and the median PFS in this group of patients was 8.5 months, which seems better than that of individuals who experienced SD among unselected NSCLC

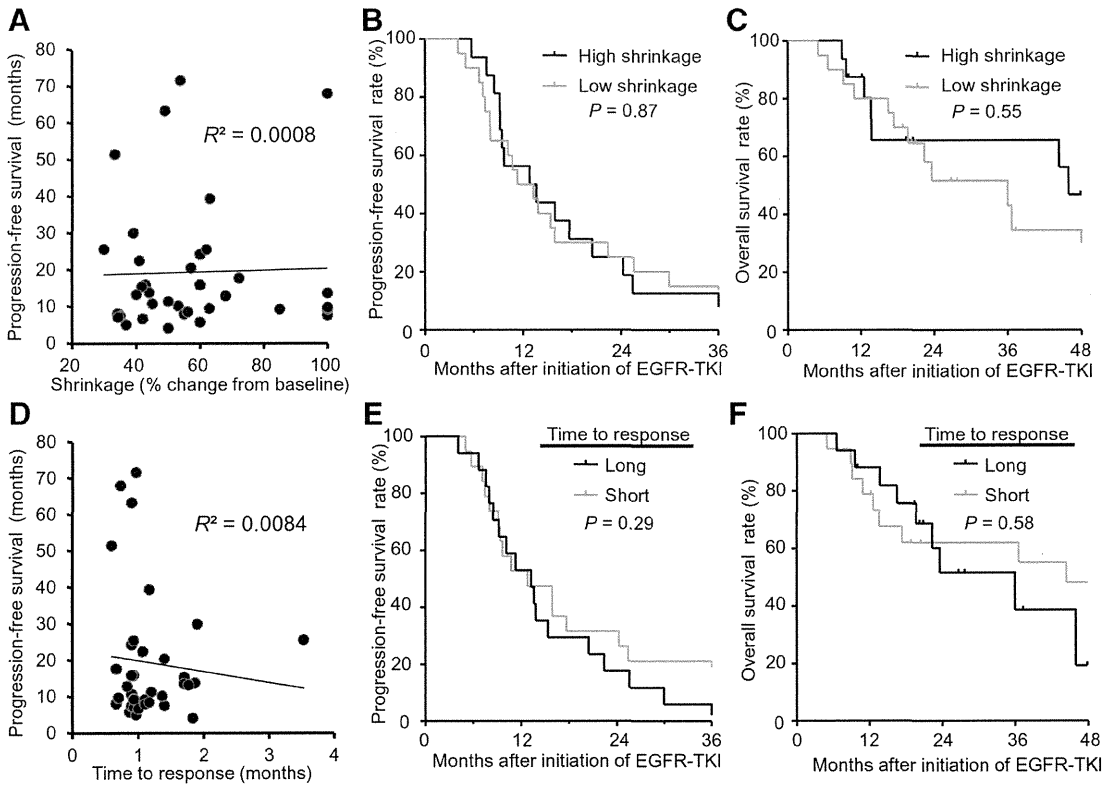


FIGURE 4. Relation between survival and either maximal tumor shrinkage or time to response for patients treated with EGFR-TKIs who achieved a complete response or partial response. *A*, Correlation between maximal tumor shrinkage and PFS. Progression-free survival (*B*) and overall survival (*C*) according to the maximal tumor shrinkage (low shrinkage, <56%; high shrinkage, ≥56%). D, Correlation between time to response and progression-free survival. Progression-free survival (*E*) and overall survival (*F*) according to the time to response (long, >4.2 weeks; short, ≤4.2 weeks). EGFR-TKI, epidermal growth factor receptor gene tyrosine kinase inhibitor.

patients treated with EGFR-TKIs.[3] Given that patients who achieved a CR or PR showed a significantly longer survival after EGFR-TKI treatment than did those who experienced SD in our study, SD might reflect an insufficient survival benefit for such treatment in *EGFR* mutation–positive patients. Further studies are therefore warranted to elucidate the molecular mechanism responsible for SD, with several candidates having been identified.[8–11] Analysis of pretreatment tumor specimens from NSCLC patients harboring *EGFR* mutations revealed that a high level of expression of hepatocyte growth factor, a ligand of the receptor tyrosine kinase MET, occurred more frequently in tumors with intrinsic EGFR-TKI resistance (SD or PD) than in sensitive tumors (PR or CR).[9] In addition, the T790M mutation of EGFR, which has been associated with acquired resistance to EGFR-TKIs in NSCLC patients harboring activating *EGFR* mutations, was recently shown to be present in some patients before treatment with EGFR-TKIs. Among *EGFR* mutation–positive NSCLC patients treated with EGFR-TKIs, those with a de novo T790M mutation were found to have a significantly shorter PFS than were those without it.[10,11] New treatment strategies are thus needed to improve outcome for patients in whom these candidate mechanisms for SD are operative.

Oncogene-addicted tumors have clinical features associated with rapid and marked tumor shrinkage after administration of a corresponding molecularly targeted drug. Such clinical features are considered to reflect early improved quality of life.[12,13] However, the impact of rapid and pronounced tumor shrinkage on survival outcome in EGFR-TKI–treated NSCLC patients who harbor an *EGFR* mutation and who show a CR or PR has remained unknown. We have now shown that neither the maximal extent of tumor shrinkage nor TTR was related to PFS or OS in such patients. Patients who harbor *EGFR* mutations eventually develop resistance to TKIs through the acquisition of additional genetic changes, such as the T790M mutation of EGFR or *MET* amplification. Our findings suggest that time to acquired resistance (disease progression) after initiation of EGFR-TKI therapy is defined by the duration of EGFR-TKI exposure, regardless of the time to onset of tumor response or the extent of tumor shrinkage.

The limitations of the present study include a retrospective design and a relatively small number of patients. Although all patients enrolled for this analysis were evaluated at least every 8 weeks until response confirmation, they underwent computed tomographic imaging at different time points.

In conclusion, response (CR or PR) may represent the optimal surrogate for survival among *EGFR* mutation–positive NSCLC patients treated with EGFR-TKIs. Moreover, our results suggest that the survival benefit of EGFR-TKI treatment in patients who achieve a CR or PR is not influenced by the pattern of tumor shrinkage.

REFERENCES

1. Sirohi B, Ashley S, Norton A, et al. Early response to platinum-based first-line chemotherapy in non-small cell lung cancer may predict survival. *J Thorac Oncol* 2007;2:735–740.
2. He L, Teng Y, Jin B, et al. Initial partial response and stable disease according to RECIST indicate similar survival for chemotherapeutical patients with advanced non-small cell lung cancer. *BMC Cancer* 2010;10:681.
3. Hotta K, Matsuo K, Ueoka H, et al. Continued gefitinib treatment after disease stabilisation prolongs survival of Japanese patients with non-small-cell lung cancer: Okayama Lung Cancer Study Group experience. *Ann Oncol* 2005;16:1817–1823.
4. Mitsudomi T, Morita S, Yatabe Y, et al.; West Japan Oncology Group. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010;11:121–128.
5. Maemondo M, Inoue A, Kobayashi K, et al.; North-East Japan Study Group. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010;362:2380–2388.
6. Zhou C, Wu YL, Chen G, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* 2011;12:735–742.
7. Rosell R, Carcereny E, Gervais R, et al.; Spanish Lung Cancer Group in collaboration with Groupe Français de Pneumo-Cancérologie and Associazione Italiana Oncologia Toracica. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* 2012;13:239–246.
8. Takeda M, Okamoto I, Fujita Y, et al. De novo resistance to epidermal growth factor receptor-tyrosine kinase inhibitors in EGFR mutation-positive patients with non-small cell lung cancer. *J Thorac Oncol* 2010;5:399–400.
9. Yano S, Yamada T, Takeuchi S, et al. Hepatocyte growth factor expression in EGFR mutant lung cancer with intrinsic and acquired resistance to tyrosine kinase inhibitors in a Japanese cohort. *J Thorac Oncol* 2011;6:2011–2017.
10. Rosell R, Molina MA, Costa C, et al. Pretreatment EGFR T790M mutation and BRCA1 mRNA expression in erlotinib-treated advanced non-small-cell lung cancer patients with EGFR mutations. *Clin Cancer Res* 2011;17:1160–1168.
11. Su KY, Chen HY, Li KC, et al. Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. *J Clin Oncol* 2012;30:433–440.
12. Thongprasert S, Duffield E, Saijo N, et al. Health-related quality-of-life in a randomized phase III first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients from Asia with advanced NSCLC (IPASS). *J Thorac Oncol* 2011;6:1872–1880.
13. Oizumi S, Kobayashi K, Inoue A, et al. Quality of life with gefitinib in patients with EGFR-mutated non-small cell lung cancer: quality of life analysis of North East Japan Study Group 002 Trial. *Oncologist* 2012;17:863–870.

Biomarkers of reactive resistance and early disease progression during chemotherapy plus bevacizumab treatment for colorectal carcinoma

Hidetoshi Hayashi[1,2,3], Tokuzo Arao[1], Kazuko Matsumoto[1], Hideharu Kimura[1], Yosuke Togashi[1], Yoshinori Hirashima[4,5], Yosuke Horita[4,6], Satoru Iwasa[4], Natsuko Tsuda Okita[4], Yoshitaka Honma[4], Atsuo Takashima[4], Ken Kato[4], Tetsuya Hamaguchi[4], Yasuhiro Shimada[4], Kazuhiko Nakagawa[2], Kazuto Nishio[1], and Yasuhide Yamada[4]

[1] Department of Genome Biology, Kinki University Faculty of Medicine, Osakasayama City, Osaka, Japan.

[2] Department of Medical Oncology, Kinki University Faculty of Medicine, Osakasayama City, Osaka, Japan.

[3] Department of Medical Oncology, Kishiwada Municipal Hospital, Kishiwada, Osaka, Japan.

[4] Gastrointestinal Medical Oncology Division, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan.

[5] Department of Medical Oncology, Oita University, Hazama-cho, Yufu, Oita, Japan.

[6] Department of Chemotherapy, Toyama Prefectural Central Hospital, Toyama, Toyama, Japan.

Correspondence to: *Hidetoshi Hayashi*, *email*: *hidet31@gmail.com*

Keywords: placental growth factor (PlGF), vascular endothelial growth factor (VEGF), colorectal carcinoma, bevacizumab, angiogenesis

Received: *January 5, 2014* *Accepted*: *March 20, 2014* *Published*: *March 22, 2014*

ABSTRACT:

Molecular markers for predicting or monitoring the efficacy of bevacizumab in patients with metastatic colorectal cancer (mCRC) remain to be identified. We have now measured the serum concentrations of 25 angiogenesis-related molecules with antibody suspension bead array systems for 25 mCRC patients both before and during treatment in a previously reported phase II trial of FOLFIRI chemotherapy plus bevacizumab. The serum concentration of vascular endothelial growth factor–A (VEGF-A) decreased after the onset of treatment ($P < 0.0001$), whereas that of placental growth factor increased ($P < 0.0001$). Significant differences in the levels of several factors (such as VEGF-A, soluble VEGF receptor–2, and interleukin-8) were apparent between responders and nonresponders during treatment. The rapid and pronounced decrease in serum VEGF-A level after treatment onset was apparent in all subjects and was independent of the baseline concentration. However, four of nine nonresponders showed a subsequent early increase in the serum VEGF-A level. Our results thus suggest that an early increase in the serum VEGF-A concentration after the initial decrease is a potential predictive marker of a poor response and reactive resistance to bevacizumab plus chemotherapy.

INTRODUCTION

Angiogenesis, defined as the formation of new blood vessels from a preexisting vasculature, is essential for tumor growth and the spread of metastases [1, 2]. Inhibition of angiogenesis is therefore considered a promising strategy for cancer treatment, with clinical application of this strategy being pursued in the form of multiple modalities that include the development of specific inhibitors of signaling by vascular endothelial growth factor (VEGF) and its cognate receptors (VEGFRs). Bevacizumab is a humanized monoclonal antibody specific for VEGF-A, a key inducer of angiogenesis in tumors, and it has been found to manifest

clinical activity in patients with metastatic colorectal cancer (mCRC) [3]. Furthermore, the cytotoxicity of chemotherapy is blunted by the production of VEGF and other proangiogenic factors that recruit new endothelial cells and protect them from chemotherapy, and bevacizumab transiently "normalizes" the abnormal structure and function of the tumor vasculature to render it more efficient for oxygen and drug delivery [4]. Indeed, bevacizumab is effective against metastatic colorectal cancer (mCRC) mainly in combination with chemotherapeutic drugs.

The efficacy of chemotherapy plus bevacizumab varies among patients, however, and so the ability to identify tumors likely to be most sensitive to such treatment would help to optimize the implementation of this approach as well as provide important insight into the mechanisms of resistance. The identification of a biomarker predictive of bevacizumab treatment outcome has proven to be challenging. Angiogenesis is a complex and highly adaptive biological process, with multiple factors in addition to VEGF-A playing an essential role, including placental growth factor (PlGF), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), angiopoietins, and various additional cytokines [5]. Reactive resistance to bevacizumab in combination with chemotherapy is mediated in part by hypoxia-inducible factor–1 (HIF-1) and its transcriptional activation of genes for multiple factors including VEGF-A and FGFs.

Extensive biomarker analysis has been conducted in numerous clinical trials of bevacizumab, with evaluation of the relation between circulating VEGF-A levels at baseline and treatment outcome having been performed in most cases [6]. Although a few studies have detected a significant correlation between the baseline serum concentration of VEGF-A and the outcome of antiangiogenic therapy [7], many others have not. The inconsistency of these results emphasizes the need for evaluation of predictive biomarkers in a dynamic manner—that is, before and after the onset of antiangiogenic treatment.

We have previously described a phase II study (AVASIRI trial) designed to investigate the efficacy of a bevacizumab plus FOLFIRI (folinic acid, 5-fluorouracil, irinotecan) regimen as a second-line treatment for individuals with metastatic colorectal cancer (mCRC) [8]. Promising results were obtained with regard to response rate (32%), progression-free survival (PFS) time (median of 11.6 months), and overall survival (OS) time (median of 21.4 months). Serum samples were collected at various time points during the trial for measurement of the levels of 25 angiogenesis-related molecules. We now present the results of the analysis of these serum samples from the AVASIRI trial.

RESULTS

Patient characteristics

Serum samples were available for all 25 patients treated with FOLFIRI and bevacizumab. The characteristics of the study patients are shown in Table 1. The median age was 62 years (range, 38–73), and the male/female distribution was 20/5. The overall response rate was 32%, with 8 patients showing a partial response, 15 stable disease, and 2 disease progression. Median progression-free survival (PFS) and overall survival (OS) were 11.6 months [95% confidence interval (CI), 6.9–16.4] and 21.4 months (95% CI, 12.0–30.8), respectively.

Circulating levels of angiogenesis-related molecules before and during treatment with FOLFIRI and bevacizumab

We examined changes in the serum concentrations of 25 angiogenesis-related molecules between before (baseline) and after the onset of treatment with FOLFIRI plus bevacizumab (Figure 1). The baseline serum concentrations varied widely among individuals, with the values for VEGF-A, for example, ranging from 13 to 907 pg/mL. Significant changes in the serum levels of various molecules were apparent at various time points during treatment compared with baseline (Figure 2). Of note, the serum concentration of VEGF-A decreased markedly after the onset of treatment (from 337.7 ± 244.4 pg/mL at baseline to 1.9 ± 5.0, 5.6 ± 12.6, 8.2 ± 17.5, and 7.3 ± 20.8 pg/mL at 1, 2, 4, and 6 months, respectively; $P < 0.0001$), whereas that of PlGF showed a pronounced increase (from 4.1 ± 3.4 pg/mL at baseline to 17.6 ± 9.0, 19.9 ± 7.9, 21.9 ± 12.3, and 24.4 ± 10.8 pg/mL at 1, 2, 4, and 6 months, respectively; $P < 0.0001$). Given that these results were obtained with paired samples from the same individuals at baseline and after the onset of treatment, the observed changes were likely attributable to the administration of FOLFIRI plus bevacizumab.

Serum concentrations of angiogenesis-related molecules and PFS

We divided the patients into two groups on the basis of progression-free survival (PFS) time. Given that the median PFS for patients with mCRC treated with chemotherapy plus bevacizumab in the second-line setting was previously found to be ~7 months [9], we dichotomized our patient population according to a PFS of 7 months (responders, ≥7 months; nonresponders, <7 months). None of the 25 molecules examined served as a predictive marker on the basis of the baseline serum

Table 1: Summary of patient characteristics and AVASIRI trial results.

Median (range) age of patients (years)	62 (38–73)
ECOG performance status 0/1	16/9
Male/female	20/5
Primary lesion in colon/rectum	12/13
Prior treatment with/without FOLFOX	16/9
Overall response rate (%)	32 (90% CI, 17.0–50.4)
Median PFS (days)	349 (95% CI, 207–491)
Median OS (days)	642 (95% CI, 359–925)

Abbreviations not defined in text: ECOG, Eastern Cooperative Oncology Group; FOLFOX, folinic acid plus 5-fluorouracil plus oxaliplatin.

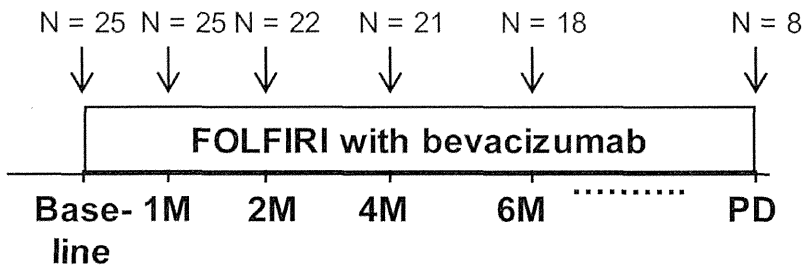


Figure 1: Flow diagram for analysis of the study subjects. Paired serum samples were available for 25 patients at baseline and at 1 month after the onset of treatment, for 22 patients at 2 months, for 21 patients at 4 months, for 18 patients at 6 months, and for 8 patients at the onset of progressive disease (PD) or last follow-up.

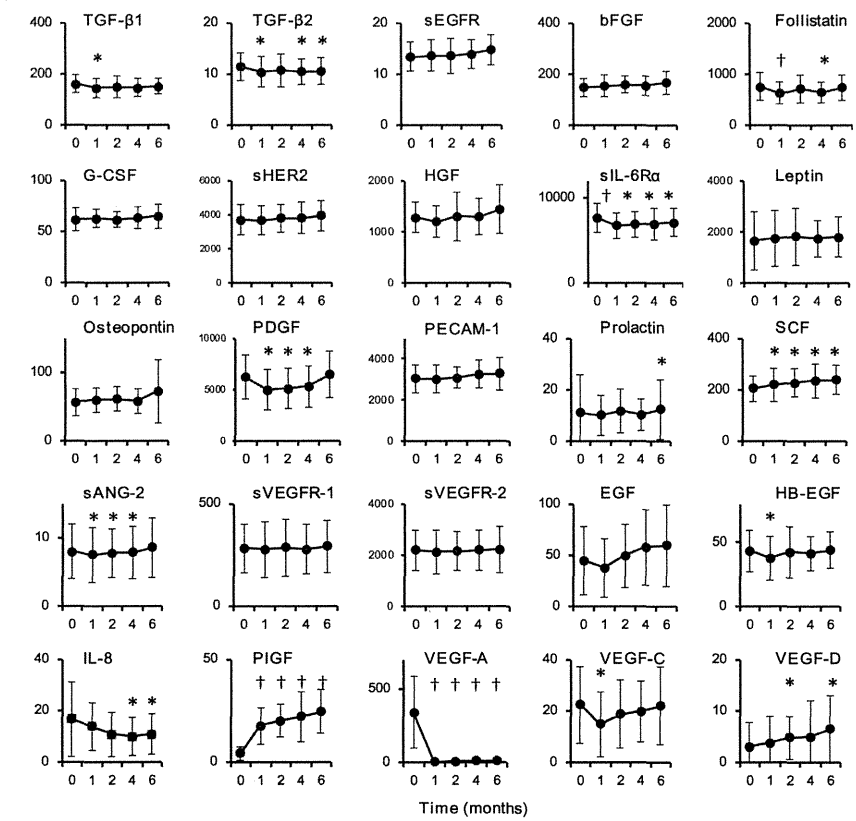


Figure 2: Serum concentrations of 25 angiogenesis-related molecules at baseline and at 1, 2, 4, and 6 months after the onset of FOLFIRI with bevacizumab treatment. Data are means ± SD for the numbers of samples indicated in Figure 1. *$P < 0.05$, †$P < 0.0001$ versus the corresponding baseline value (Student's paired t test). All values represent picograms per milliliter.

concentrations, whereas significant differences in the levels of various molecules [including soluble VEGFR-2 (sVEGFR-2), interleukin (IL)–8, VEGF-A, and VEGF-C] at various time points during treatment were apparent between responders and nonresponders (Table 2).

Relation between FOLFIRI-bevacizumab treatment and changes in serum VEGF-A level

Finally, we investigated the relation between changes in the serum concentration of VEGF-A and the duration of treatment with FOLFIRI plus bevacizumab (Figure 3). Several patients manifested an increase in the serum VEGF-A level around the time of disease progression relative to the lowered value apparent after the onset of treatment and during its administration for several months. Of note, four nonresponders showed an early increase in the serum concentration of VEGF-A (cases 17–20 in Figure 3B). The PFS of these four patients was significantly shorter than that of the other 21 patients (200 versus 373 days, respectively; $P = 0.009$, Student's unpaired t test), suggesting that an early increase in serum VEGF-A level subsequent to an initial decrease is predictive of early resistance to bevacizumab. On the other hand, the serum concentration of VEGF-A remained stable at the time of disease progression in other patients (cases 13–16). Patient 15 continued treatment with bevacizumab, in combination with a different chemotherapy regimen (mFOLFOX6), beyond disease progression.

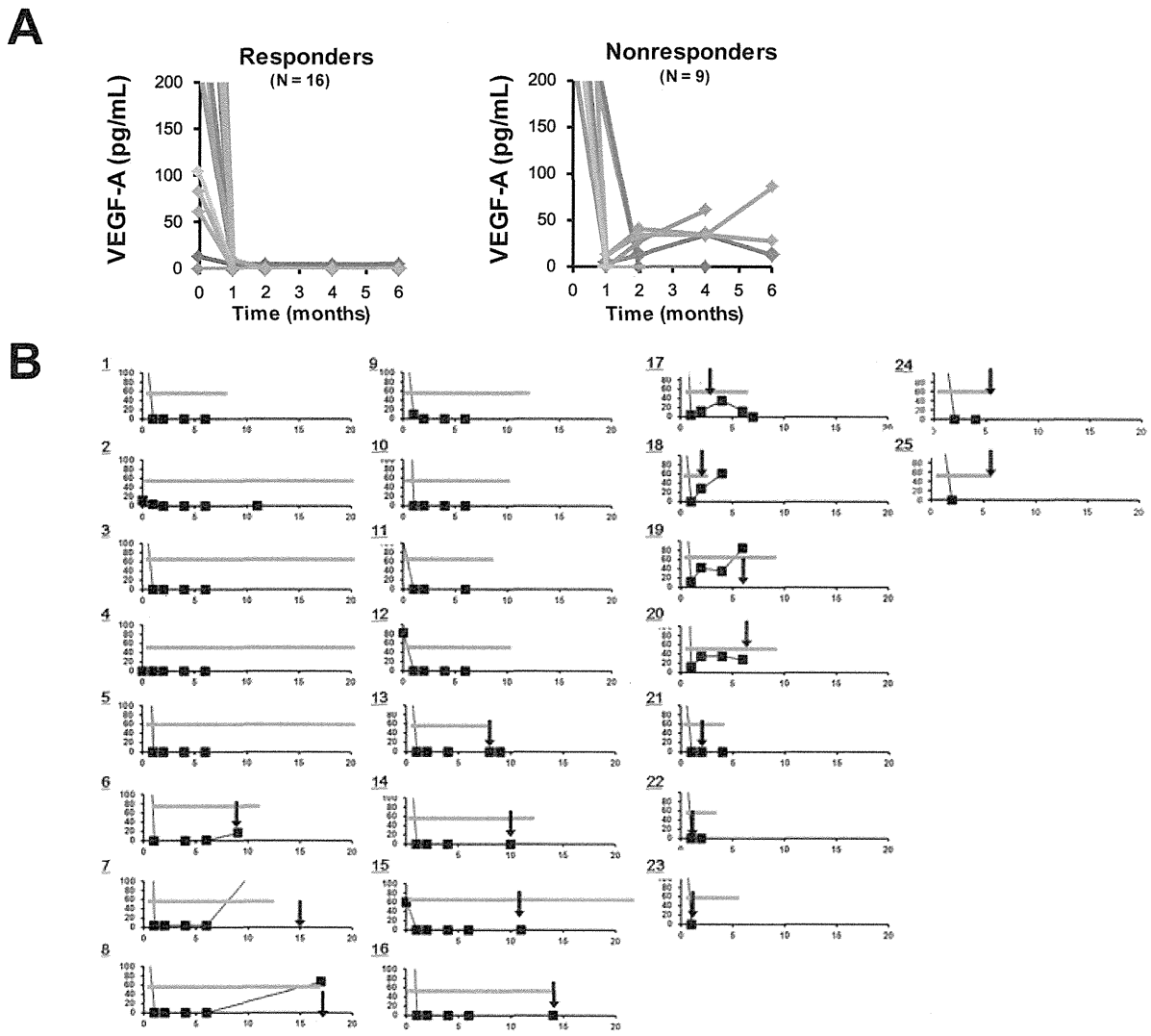


Figure 3: Analysis of changes in the serum concentration of VEGF-A. (A) Time course of serum VEGF-A level in responders and nonresponders. (B) Time course of serum VEGF-A concentration in relation to the duration of treatment with bevacizumab plus chemotherapy (gray bars) and the detection of disease progression (black arrows). Cases 1 to 16 and 17 to 25 correspond to responders and nonresponders, respectively. The vertical and horizontal axes represent serum VEGF-A (pg/mL) and time (months), respectively.

Table 2: Serum concentrations of 25 angiogenesis-related molecules at baseline and at the indicated times after the onset of treatment with FOLFIRI plus bevacizumab in responders and nonresponders. Data are means ± SD. The P values for comparisons between responders (RES) and nonresponders (non-RES) were determined with Student's unpaired t test; those of <0.05 are shown in bold.

	Serum concentration (pg/ml)														
	Baseline (N = 25)			1 month (N = 25)			2 months (N = 22)			4 months (N = 21)			6 months (N = 18)		
	RES	non-RES	*P* value	RES	non-RES	*P* value	RES	non-RES	*P* value	RES	non-RES	*P* value	RES	non-RES	*P* value
TGF-β1	164 ± 33	149 ± 46	0.443	145 ± 39	138 ± 46	0.732	142 ± 32	159 ± 70	0.594	151 ± 35	121 ± 26	0.067	157 ± 29	123 ± 28	0.086
TGF-β2	12 ± 3	12 ± 3	0.818	10 ± 3	10 ± 3	0.961	11 ± 4	12 ± 2	0.379	11 ± 3	10 ± 1	0.362	11 ± 3	9 ± 1	0.176
sEGFR	13 ±3	14 ± 4	0.838	13 ± 3	14 ± 4	0.571	13 ± 3	15 ± 4	0.241	13 ± 3	16 ± 3	0.190	14 ± 3	18 ± 2	**0.018**
bFGF	146 ± 38	159 ± 26	0.352	150 ± 47	167 ± 33	0.346	149 ± 35	182 ± 25	**0.031**	149 ± 40	177 ± 29	0.115	157 ± 43	195 ± 39	0.156
Follistatin	736 ± 310	766 ± 198	0.783	627 ± 257	603 ± 143	0.778	664 ± 206	860 ± v388	0.284	620 ± 202	745 ± 185	0.242	655 ± 227	997 ± 131	**0.004**
G-CSF	61 ± 11	64 ± 10	0.610	63 ± 10	62 ± 8	0.947	62 ± 7	60 ± 11	0.719	65 ± 10	59 ± 12	0.347	64 ± 10	65 ± 19	0.966
sHER2	3697 ± 920	3914 ± 811	0.580	3645 ± 971	3906 ± 599	0.443	3615 ± 917	4207 ± 306	**0.040**	3737 ± 840	4148 ± 1011	0.443	3700 ± 856	4686 ± 429	**0.010**
HGF	1222 ± 279	1470 ± 303	0.093	1155 ± 325	1332 ± 289	0.216	1157 ± 293	1660 ± 674	0.131	1201 ± 274	1599 ± 422	0.103	1289 ± 362	1921 ± 573	0.110
sIL-6Rα	7883 ± 1714	6899 ± 1465	0.183	7037 ± 1562	5905 ± 825	**0.035**	6981 ± 1672	6097 ± 700	0.104	7020 ± 1616	6266 ± 2505	0.555	7134 ± 1715	6816 ± 1467	0.727
Leptin	1608 ± 1048	2038 ± 1364	0.475	1704 ± 1045	2119 ± 1237	0.457	1765 ± 912	2054 ± 1592	0.690	1823 ± 702	1625 ± 805	0.640	1903 ± 836	1379 ± 455	0.133
Osteopontin	54 ± 21	59 ± 18	0.582	58 ± 20	61 ± 16	0.665	57 ± 17	67 ± 21	0.366	54 ± 12	67 ± 32	0.441	65 ± 31	95 ± 86	0.538
PDGF	6297 ± 2439	6488 ± 1321	0.812	4978 ± 2276	5095 ± 1460	0.884	4770 ± 2124	5874 ± 1753	0.246	5224 ± 2244	5372 ± 1825	0.885	6095 ± 2085	7582 ± 2702	0.365
PECAM-1	3034 ± 749	3116 ± 469	0.753	3018 ± 746	3081 ± 548	0.823	2959 ± 544	3294 ± 257	0.072	3211 ± 785	3468 ± 323	0.317	3097 ± 760	3857 ± 711	0.121
Prolactin	10 ± 17	10 ± 5	0.964	9 ± 9	11 ± 5	0.451	11 ± 9	15 ± 9	0.374	10 ± 6	13 ± 8	0.450	8 ± 4	26 ± 19	0.159
SCF	207 ± 55	210 ± 44	0.892	218 ± 62	235 ± 71	0.587	218 ± 55	249 ± 55	0.270	226 ± 55	276 ± 91	0.297	227 ± 61	279 ± 20	**0.016**
sANG-2	8 ± 4	10 ± 3	0.139	7 ± 4	9 ± 3	0.292	7 ± 4	10 ± 2	**0.030**	7 ± 4	11 ± 3	**0.027**	8 ± 4	12 ± 3	0.062
sVEGFR-1	289 ± 139	289 ± 67	0.997	282 ± 166	268 ± 67	0.774	261 ± 133	358 ± 148	0.199	264 ± 123	324 ± 120	0.366	275 ± 140	344 ± 68	0.201
sVEGFR-2	2128 ± 873	2388 ± 662	0.445	2067 ± 917	2338 ± 785	0.483	2001 ± 750	2567 ± 799	0.171	1995 ± 792	2794 ± 418	**0.012**	2004 ± 887	2936 ± 473	**0.020**
EGF	50 ± 34	26 ± 28	0.097	38 ± 30	31 ± 28	0.628	58 ± 32	31 ± 21	**0.038**	59 ± 39	56 ± 37	0.861	59 ± 40	62 ± 43	0.882
HB-EGF	44 ± 14	42 ± 24	0.897	39 ± 13	38 ± 26	0.966	40 ± 13	48 ± 32	0.573	43 ± 14	35 ± 9	0.138	46 ± 15	35 ± 12	0.183
IL-8	14 ± 15	24 ± 13	0.115	10 ± 6	19 ± 7	**0.027**	8 ± 6	17 ± 11	0.109	8 ± 6	15 ± 10	0.216	9 ± 6	18 ± 10	0.165
PIGF	4 ± 3	5 ± 4	0.635	17 ± 9	20 ± 9	0.473	19 ± 8	19 ± 6	0.878	18 ± 7	34 ± 18	0.127	22 ± 8	33 ± 15	0.246
VEGF-A	333 ± 295	351 ± 143	0.842	1 ± 3	4 ± 6	0.228	0 ± 1	20 ± 18	**0.046**	0 ± 1	33 ± 22	**0.027**	0 ± 1	42 ± 38	0.198
VEGF-C	23 ± 16	23 ± 16	0.995	15 ± 15	16 ± 7	0.813	16 ± 13	27 ± 12	0.082	17 ± 12	28 ± 8	**0.047**	21 ± 16	25 ± 11	0.603
VEGF-D	3 ± 3	1 ± 2	0.235	4 ± 4	1 ± 2	0.083	5 ± 4	4 ± 6	0.626	5 ± 8	6 ± 5	0.713	6 ± 6	7 ± 8	0.765

DISCUSSION

The introduction of novel molecularly targeted therapies, including antiangiogenic and anti–epidermal growth factor receptor (EGFR) agents, has increased the options available for treatment of mCRC [9]. At present, bevacizumab in combination with fluoropyrimidine-based chemotherapy is widely recognized as a standard treatment for mCRC [3, 9, 10]. However, no biomarker has previously been identified as a predictor of benefit from bevacizumab treatment, with the identification of such a molecular biomarker being a current priority of clinical research [11]. In the present study, we have addressed this issue by measuring the serum levels of multiple angiogenesis-related factors both before and during treatment of mCRC patients with bevacizumab plus FOLFIRI.

Most previous studies have found that the circulating concentration of VEGF-A, as measured with standard enzyme-linked immunosorbent assays, increases after the onset of antiangiogenic treatment [7, 12-18], whereas more recent studies have shown a decrease in VEGF-A levels after treatment onset [19-21]. We have now shown that treatment with bevacizumab plus chemotherapy was associated with a rapid and highly significant decrease in the serum concentration of VEGF-A that was independent of the baseline concentration and which, in most cases, remained apparent throughout the duration of therapy, similar to the results of a previous pharmacodynamic analysis of angiogenesis-related factors [22]. Although it remains unclear whether circulating VEGF-A in individuals treated with bevacizumab is free or bound to the antibody, given that bevacizumab is administered at doses high enough to give rise to such binding, our results suggest that the antibody suspension bead array system adopted in the present study measures VEGF-A that is free of bevacizumab.

An initial decrease in serum VEGF-A level was observed in all patients of the present study. However, some patients manifested a subsequent early small but definite increase in this parameter. This latter finding may be related to the assay measuring free VEGF-A and may therefore reflect a compensatory increase in the circulating concentration of this factor. Our observation that the PFS of such patients was shorter than that of the other subjects suggests that the development of acquired resistance to bevacizumab treatment may be driven in part by loss of the ability to suppress the circulating level of free VEGF-A. VEGF-A promotes the survival of and increases resistance to chemotherapy in cancer cells. Chemotherapy acts as an "accidental" antiangiogenic therapy (action), whereas VEGF-A and other proangiogenic factors recruit new endothelial cells and protect them from the cytotoxicity of chemotherapy (reaction) [4, 23]. Bevacizumab is thought to block this reaction. From this perspective, our results suggest that an early increase in VEGF-A levels after the initial decrease is a potential predictive marker of reactive resistance to bevacizumab that results in a shorter PFS in patients treated with the combination of FOLFIRI and bevacizumab.

Despite the predominant role of VEGF-A, multiple other factors contribute to regulation of the complex and highly adaptive process of angiogenesis. Investigation of potential biomarkers other than VEGF-A is thus important, given the role of these other factors in tumor angiogenesis and vessel maturation. However, only a few studies have previously examined multiple angiogenesis-related proteins during bevacizumab treatment in a dynamic

manner [22]. We have now detected significant treatment-induced changes in the serum concentrations of several angiogenesis-related molecules including PlGF. Previous biomarker analyses also described an increase in the circulating concentration of PlGF in response to VEGF-targeted treatment [12, 14, 15, 17, 22]. Indeed, targeting of PlGF is under consideration as a novel approach to prevent tumor escape from VEGF-targeted therapy [24]. However, we did not detect a significant difference in serum PlGF levels after bevacizumab administration between responders and nonresponders in the present study. A previous study also found that the combination of antibodies to PlGF and those to VEGF-A did not yield a greater antitumor effect in vitro or in vivo compared with antibodies to VEGF-A alone [25]. Our data thus suggest that the increase in circulating PlGF level observed after the onset of bevacizumab treatment does not play a major role in the development of resistance to bevacizumab in the clinical setting.

On the other hand, we detected significantly higher serum concentrations of several angiogenesis-related factors [such as IL-8, soluble angiopoietin II (sANG-2), basic FGF (bFGF), stem cell factor (SCF), and VEGF-C] in nonresponders compared with responders at various time points during treatment. Resistance to VEGF-A pathway inhibitors might occur through VEGF-A–independent mechanisms, such as up-regulation of other proangiogenic factors [26-28]. Given that targeting of these molecules may provide a basis for novel approaches to prevent tumor escape from bevacizumab treatment, further analysis of multiple angiogenesis-related factors in a large number of patients is warranted.

In conclusion, our present results indicate that an early increase in the serum concentration of VEGF-A after the initial decrease may be a potential predictive marker of a poor response and reactive resistance to bevacizumab plus chemotherapy.

METHODS

Patients

The main inclusion criteria for the present study were the same as those previously described for the AVASIRI trial [8]. In brief, they comprised a histologically confirmed diagnosis of colorectal cancer; failure of first-line treatment with 5-fluorouracil– or oxaliplatin-based chemotherapy without bevacizumab or CPT-11 (irinotecan); measurable disease according to RECIST (ver. 1.0); and metastatic disease deemed unresectable at baseline. Enrolled patients received biweekly administrations of the FOLFIRI regimen, consisting of CPT-11 (150 mg/m^2) on day 1, given as a 2-h infusion concurrent with leucovorin (folinic acid, 200 mg/m^2), followed by 5-fluorouracil given by injection (400 mg/m^2) and then as a 46-h continuous infusion (2400 mg/m^2). Bevacizumab was administered at a biweekly dose of 10 mg/kg before the FOLFIRI regimen. Treatment was discontinued in the event of disease progression, unacceptable toxicity, or withdrawal of consent. Patients underwent a computed tomography scan after every four cycles of treatment for evaluation of tumor response. They provided written informed consent to receive the treatment and to participate in translational analyses.

Sample collection and analysis

Blood samples were obtained from all assigned patients at baseline (before the first dose of study drugs) as well as at 1, 2, 4, and 6 months after the onset of the treatment protocol (Figure 1). In addition, blood samples from eight patients who received the study treatment for >6 months were obtained at the time of disease progression or last follow-up. Serum separated from the blood samples was stored at –80°C until analysis.

The serum levels of VEGF-A, VEGF-C, VEGF-D, PlGF, epidermal growth factor (EGF), IL-8, and heparin-binding EGF-like growth factor (HB-EGF) were measured with the use of a Milliplex MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel (Merck Millipore, Billerica, MA, USA). Magnetic antibody-conjugated beads were subjected to ultrasonic treatment for 30 s and then to vortex-mixing for 1 min in order to reduce bead aggregation. All samples, quality controls, and standards were prepared as recommended with the supplied diluents and were processed in duplicate batches. Assay buffer (200 μL) was added to each well and then decanted. Each sample (25 μL) and the prepared beads (25 μL) were then added to the wells together with buffering solutions. The plate was sealed, incubated overnight at 4°C, and washed three times, after which detection antibodies (25 μL) were added to each well and the plate was incubated for 1 h at room temperature. Streptavidin-phycoerythrin (25 μL) was then added to each well, after which the plate was incubated for an additional 30 min at room temperature and washed three times. Sheath fluid (100 μL) was finally added to each well, and the assay plate was analyzed with the Luminex 100 instrument.

The serum levels of transforming growth factor (TGF)–β1 and TGF-β2 were measured with a Milliplex MAP Multi-Species TGFβ 3-Plex panel (Merck Millipore), whereas those of various additional factors related to angiogenesis were measured with a Bio-Plex Pro Human Cancer Biomarker Panel 1, 16-Plex (Bio-Rad, Hercules, CA, USA) as previously described [29]. The latter factors included soluble EGFR (sEGFR), bFGF, osteopontin, PDGF–AB/BB, follistatin, granulocyte colony-stimulating factor (G-CSF), platelet endothelial cell adhesion molecule–1 (PECAM-1), prolactin, soluble human EGF receptor 2/NEU (sHER2/NEU), hepatocyte

growth factor (HGF), SCF, sANG-2, soluble IL-6 receptor α (sIL-6Rα), leptin, sVEGFR-1, and sVEGFR-2.

Statistical analysis

Serum factor levels at baseline (pretreatment) were compared with those at 1, 2, 4, or 6 months after treatment onset with the use of Student's paired *t* test in order to evaluate the significance of changes induced by the study treatment. The relations between treatment efficacy and serum factor levels were analyzed with Student's unpaired *t* test. A *P* value of <0.05 was considered statistically significant. All statistical tests were performed with SPSS version 14.0 software (SPSS, Chicago, IL, USA).

ACKNOWLEDGMENTS

We thank Shinji Kurashimo, Yoshihiro Mine, Ayaka Kurumatani, and Tomoko Kitayama for technical assistance. This study was supported by The Third-Term Comprehensive 10-Year Strategy for Cancer Control of, and by a Grant-in-Aid for Cancer Research from, the Ministry of Health, Labor, and Welfare of Japan. The authors declare no conflicts of interest.

REFERENCES

1. Taylor M, Rossler J, Geoerger B, Laplanche A, Hartmann O, Vassal G, Farace F. High levels of circulating VEGFR2+ Bone marrow-derived progenitor cells correlate with metastatic disease in patients with pediatric solid malignancies. Clin Cancer Res 2009; 15: 4561-4571.
2. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2002; 2: 727-739.
3. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350: 2335-2342.
4. Blagosklonny MV. How Avastin potentiates chemotherapeutic drugs: action and reaction in antiangiogenic therapy. Cancer Biol Ther 2005; 4: 1307-1310.
5. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature 2011; 473: 298-307.
6. Lambrechts D, Lenz HJ, de Haas S, Carmeliet P, Scherer SJ. Markers of response for the antiangiogenic agent bevacizumab. J Clin Oncol 2013; 31: 1219-1230.
7. Burstein HJ, Chen YH, Parker LM, Savoie J, Younger J, Kuter I, Ryan PD, Garber JE, Chen H, Campos SM, Shulman LN, Harris LN, Gelman R, Winer EP. VEGF as a marker for outcome among advanced breast cancer patients receiving anti-VEGF therapy with bevacizumab and vinorelbine chemotherapy. Clin Cancer Res 2008; 14: 7871-7877.
8. Horita Y, Yamada Y, Kato K, Hirashima Y, Akiyoshi K, Nagashima K, Nakajima T, Hamaguchi T, Shimada Y. Phase II clinical trial of second-line FOLFIRI plus bevacizumab for patients with metastatic colorectal cancer: AVASIRI trial. Int J Clin Oncol 2012; 17: 604-609.
9. Giantonio BJ, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, Schwartz MA, Benson AB, 3rd. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. J Clin Oncol 2007; 25: 1539-1544.
10. Cassidy J, Clarke S, Diaz-Rubio E, Scheithauer W, Figer A, Wong R, Koski S, Lichinitser M, Yang TS, Rivera F, Couture F, Sirzen F, Saltz L. Randomized phase III study of capecitabine plus oxaliplatin compared with fluorouracil/folinic acid plus oxaliplatin as first-line therapy for metastatic colorectal cancer. J Clin Oncol 2008; 26: 2006-2012.
11. Shaked Y, Bocci G, Munoz R, Man S, Ebos JM, Hicklin DJ, Bertolini F, D'Amato R, Kerbel RS. Cellular and molecular surrogate markers to monitor targeted and non-targeted antiangiogenic drug activity and determine optimal biologic dose. Curr Cancer Drug Targets 2005; 5: 551-559.
12. Batchelor TT, Sorensen AG, di Tomaso E, Zhang WT, Duda DG, Cohen KS, Kozak KR, Cahill DP, Chen PJ, Zhu M, Ancukiewicz M, Mrugala MM, Plotkin S, Drappatz J, Louis DN, Ivy P, Scadden DT, Benner T, Loeffler JS, Wen PY, Jain RK. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell 2007; 11: 83-95.
13. Norden-Zfoni A, Desai J, Manola J, Beaudry P, Force J, Maki R, Folkman J, Bello C, Baum C, DePrimo SE, Shalinsky DR, Demetri GD, Heymach JV. Blood-based biomarkers of SU11248 activity and clinical outcome in patients with metastatic imatinib-resistant gastrointestinal stromal tumor. Clin Cancer Res 2007; 13: 2643-2650.
14. Zhu AX, Sahani DV, Duda DG, di Tomaso E, Ancukiewicz M, Catalano OA, Sindhwani V, Blaszkowsky LS, Yoon SS, Lahdenranta J, Bhargava P, Meyerhardt J, Clark JW, Kwak EL, Hezel AF, Miksad R, Abrams TA, Enzinger PC, Fuchs CS, Ryan DP, Jain RK. Efficacy, safety, and potential biomarkers of sunitinib monotherapy in advanced hepatocellular carcinoma: a phase II study. J Clin Oncol 2009; 27: 3027-3035.
15. Willett CG, Duda DG, di Tomaso E, Boucher Y, Ancukiewicz M, Sahani DV, Lahdenranta J, Chung DC, Fischman AJ, Lauwers GY, Shellito P, Czito BG, Wong TZ, Paulson E, Poleski M, Vujaskovic Z, Bentley R, Chen HX, Clark JW, Jain RK. Efficacy, safety, and biomarkers of neoadjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer: a multidisciplinary phase II

study. J Clin Oncol 2009; 27: 3020-3026.

16. Willett CG, Boucher Y, Duda DG, di Tomaso E, Munn LL, Tong RT, Kozin SV, Petit L, Jain RK, Chung DC, Sahani DV, Kalva SP, Cohen KS, Scadden DT, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Shellito PC, Mino-Kenudson M, Lauwers GY. Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. J Clin Oncol 2005; 23: 8136-8139.
17. Rini BI, Michaelson MD, Rosenberg JE, Bukowski RM, Sosman JA, Stadler WM, Hutson TE, Margolin K, Harmon CS, DePrimo SE, Kim ST, Chen I, George DJ. Antitumor activity and biomarker analysis of sunitinib in patients with bevacizumab-refractory metastatic renal cell carcinoma. J Clin Oncol 2008; 26: 3743-3748.
18. Saltz LB, Rosen LS, Marshall JL, Belt RJ, Hurwitz HI, Eckhardt SG, Bergsland EK, Haller DG, Lockhart AC, Rocha Lima CM, Huang X, DePrimo SE, Chow-Maneval E, Chao RC, Lenz HJ. Phase II trial of sunitinib in patients with metastatic colorectal cancer after failure of standard therapy. J Clin Oncol 2007; 25: 4793-4799.
19. Del Vecchio M, Mortarini R, Canova S, Di Guardo L, Pimpinelli N, Sertoli MR, Bedognetti D, Queirolo P, Morosini P, Perrone T, Bajetta E, Anichini A. Bevacizumab plus fotemustine as first-line treatment in metastatic melanoma patients: clinical activity and modulation of angiogenesis and lymphangiogenesis factors. Clin Cancer Res 2010; 16: 5862-5872.
20. Pectasides D, Papaxoinis G, Kalogeras KT, Eleftheraki AG, Xanthakis I, Makatsoris T, Samantas E, Varthalitis I, Papakostas P, Nikitas N, Papandreou CN, Pentheroudakis G, Timotheadou E, Koutras A, Sgouros J, Bafaloukos D, Klouvas G, Economopoulos T, Syrigos KN, Fountzilas G. XELIRI-bevacizumab versus FOLFIRI-bevacizumab as first-line treatment in patients with metastatic colorectal cancer: a Hellenic Cooperative Oncology Group phase III trial with collateral biomarker analysis. BMC Cancer 2012; 12: 271.
21. Karihtala P, Maenpaa J, Turpeenniemi-Hujanen T, Puistola U. Front-line bevacizumab in serous epithelial ovarian cancer: biomarker analysis of the FINAVAST trial. Anticancer Res 2010; 30: 1001-1006.
22. Loupakis F, Cremolini C, Fioravanti A, Orlandi P, Salvatore L, Masi G, Di Desidero T, Canu B, Schirripa M, Frumento P, Di Paolo A, Danesi R, Falcone A, Bocci G. Pharmacodynamic and pharmacogenetic angiogenesis-related markers of first-line FOLFOXIRI plus bevacizumab schedule in metastatic colorectal cancer. Br J Cancer 2011; 104: 1262-1269.
23. Blagosklonny MV. Antiangiogenic therapy and tumor progression. Cancer Cell 2004; 5: 13-17.
24. Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, Chorianopoulos E, Liesenborghs L, Koch M, De Mol M, Autiero M, Wyns S, Plaisance S, Moons L, van Rooijen N, Giacca M, Stassen JM, Dewerchin M, Collen D, Carmeliet P. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell 2007; 131: 463-475.
25. Bais C, Wu X, Yao J, Yang S, Crawford Y, McCutcheon K, Tan C, Kolumam G, Vernes JM, Eastham-Anderson J, Haughney P, Kowanetz M, Hagenbeek T, Kasman I, Reslan HB, Ross J, Van Bruggen N, Carano RA, Meng YJ, Hongo JA, Stephan JP, Shibuya M, Ferrara N. PlGF blockade does not inhibit angiogenesis during primary tumor growth. Cell 2010; 141: 166-177.
26. Lieu CH, Tran H, Jiang ZQ, Mao M, Overman MJ, Lin E, Eng C, Morris J, Ellis L, Heymach JV, Kopetz S. The Association of Alternate VEGF Ligands with Resistance to Anti-VEGF Therapy in Metastatic Colorectal Cancer. PLoS One 2013; 8: e77117.
27. Crawford Y, Kasman I, Yu L, Zhong C, Wu X, Modrusan Z, Kaminker J, Ferrara N. PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. Cancer Cell 2009; 15: 21-34.
28. Ferrara N. Pathways mediating VEGF-independent tumor angiogenesis. Cytokine Growth Factor Rev 2010; 21: 21-26.
29. Li D, Chiu H, Gupta V, Chan DW. Validation of a multiplex immunoassay for serum angiogenic factors as biomarkers for aggressive prostate cancer. Clin Chim Acta 2012; 413: 1506-1511.

Published OnlineFirst June 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1559

Cancer Therapy: Preclinical

Clinical Cancer Research

Tyrosine Phosphoproteomics Identifies Both Codrivers and Cotargeting Strategies for T790M-Related EGFR-TKI Resistance in Non–Small Cell Lung Cancer

Takeshi Yoshida[1,5], Guolin Zhang[1], Matthew A. Smith[1], Alex S. Lopez[2], Yun Bai[1], Jiannong Li[1], Bin Fang[3], John Koomen[3], Bhupendra Rawal[4], Kate J. Fisher[4], Ann Y. Chen[4], Michiko Kitano[5], Yume Morita[5], Haruka Yamaguchi[5], Kiyoko Shibata[5], Takafumi Okabe[5], Isamu Okamoto[5], Kazuhiko Nakagawa[5], and Eric B. Haura[1]

Abstract

Purpose: Irreversible EGFR-tyrosine kinase inhibitors (TKI) are thought to be one strategy to overcome EGFR-TKI resistance induced by T790M gatekeeper mutations in non–small cell lung cancer (NSCLC), yet they display limited clinical efficacy. We hypothesized that additional resistance mechanisms that cooperate with T790M could be identified by profiling tyrosine phosphorylation in NSCLC cells with acquired resistance to reversible EGFR-TKI and harboring T790M.

Experimental Design: We profiled PC9 cells with TKI-sensitive *EGFR* mutation and paired EGFR-TKI–resistant PC9GR (gefitinib-resistant) cells with T790M using immunoaffinity purification of tyrosine-phosphorylated peptides and mass spectrometry–based identification/quantification. Profiles of erlotinib perturbations were examined.

Results: We observed a large fraction of the tyrosine phosphoproteome was more abundant in PC9- and PC9GR-erlotinib–treated cells, including phosphopeptides corresponding to MET, IGF, and AXL signaling. Activation of these receptor tyrosine kinases by growth factors could protect PC9GR cells against the irreversible EGFR-TKI afatinib. We identified a Src family kinase (SFK) network as EGFR-independent and confirmed that neither erlotinib nor afatinib affected Src phosphorylation at the activation site. The SFK inhibitor dasatinib plus afatinib abolished Src phosphorylation and completely suppressed downstream phosphorylated Akt and Erk. Dasatinib further enhanced antitumor activity of afatinib or T790M-selective EGFR-TKI (WZ4006) in proliferation and apoptosis assays in multiple NSCLC cell lines with T790M-mediated resistance. This translated into tumor regression in PC9GR xenograft studies with combined afatinib and dasatinib.

Conclusions: Our results identified both codrivers of resistance along with T790M and support further studies of irreversible or T790M-selective EGFR inhibitors combined with dasatinib in patients with NSCLC with acquired T790M. *Clin Cancer Res; 20(15); 4059–74. ©2014 AACR.*

Introduction

Despite the benefits shown with EGFR-tyrosine kinase inhibitor (EGFR-TKI) treatment in patients with non–small cell lung cancer (NSCLC) with TKI-sensitive *EGFR* mutations (1, 2), acquired resistance is a critical clinical problem. A secondary point mutation in exon 20 of *EGFR* that substitutes methionine for threonine at amino acid position 790 (T790M) was identified in patients with NSCLC who developed acquired resistance to gefitinib or erlotinib (3, 4). Nearly 50% of patients with NSCLC with acquired resistance to EGFR-TKIs have the T790M secondary mutation (5–7). Irreversible EGFR-TKIs, such as CL387,785 (8), PF00299804 (9), BIBW-2992 (afatinib; ref. 10), and HKI-272 (11), are thought to be one strategy to overcome T790M-induced resistance. However, a number of studies have shown their limited activity in cells with T790M mutations given the increased affinity of ATP binding to T790M EGFR proteins or through mechanisms affecting other pathways such as MET activation (8, 9, 12–18). Clinical studies have also highlighted the limited efficacy of irreversible EGFR-TKIs. In the LUX-Lung 1 Trial,

Authors' Affiliations: [1]Department of Thoracic Oncology, [2]Tissue Core, [3]Proteomics and Molecular Oncology Program, [4]Biostatistics Program, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida; and [5]Department of Medical Oncology, Kinki University Faculty of Medicine, Osaka-Sayama, Osaka, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

T. Yoshida and G. Zhang contributed equally to this article.

Corresponding Author: Eric B. Haura, Department of Thoracic Oncology, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612. Phone: 181-3745-6827; Fax: 181-3745-6817; E-mail: eric.haura@moffitt.org

doi: 10.1158/1078-0432.CCR-13-1559

Published OnlineFirst June 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1559

Translational Relevance

Acquired resistance to EGFR-tyrosine kinase inhibitor (EGFR-TKI) is a critical clinical problem in patients with non–small cell lung cancer (NSCLC) with TKI-sensitive *EGFR* mutation. We applied mass spectrometry–based tyrosine phosphoproteomics to paired TKI-sensitive and resistant cell lines to visualize molecular networks related to the acquired EGFR-TKI resistance. The results suggest that multiple receptors and signaling molecules such as MET, AXL, and IRS2 can collaborate to drive resistance to EGFR-TKI. We also identified Src family kinases (SFK) as a central signaling hub in TKI-resistant cells with T790M gatekeeper mutation. SFK phosphorylation was also detected in human NSCLC samples with T790M. *In vitro* and *in vivo* experiments demonstrated that irreversible EGFR-TKI (afatinib) or T790M-selective EGFR-TKI (WZ4006) combined with the SFK inhibitor dasatinib overcame T790M-mediated resistance, thereby nominating a new strategy for translation into the clinic.

conducted to compare afatinib treatment versus placebo in patients with advanced NSCLC whose disease progressed after receiving first-generation EGFR-TKIs (erlotinib, gefitinib), afatinib did not extend the primary endpoint of overall survival despite significant improvements in progression-free survival (19). These preclinical and clinical results suggest that irreversible EGFR-TKIs as single agents are insufficient to overcome resistance.

One strategy to improve on the limited efficacy of irreversible EGFR-TKI is through combination with other pathway inhibitors. For example, studies that combined afatinib with the anti-EGFR monoclonal antibody cetuximab (20) or the PI3K/mTOR inhibitor PI-103 (12) and HKI-272 combined with mTOR inhibitor rapamycin (21) have shown promise in overcoming T790M resistance. Another reason for the limited efficacy of agents targeting T790M could be mediated through other tyrosine kinases, such as receptor tyrosine kinases (RTK), which provide additional protection against EGFR-TKIs (22). Recent studies have shown that growth factor ligands can protect oncogene-addicted cells from molecularly targeted agents; thus, altered expression of these growth factor receptors could further identify resistance pathways (23–25).

We explored the underlying ability of some growth factor ligands to drive resistance to TKIs by examining the basal tyrosine phosphoproteome and the effects of EGFR-TKIs on other RTKs. In this study, we tested the hypothesis that a global evaluation of tyrosine phosphorylation (using mass spectrometry) between the sensitive and resistant cells, along with EGFR perturbations, could identify additional resistance mechanisms that could give insight into cotargeting strategies. Our results identified numerous coexpressed RTKs and non-RTKs that, under proper environmental circumstances, cooperate to drive resistance to EGFR-TKIs. We further showed that Src family kinase (SFK) signaling was independent of EGFR signaling and that cotargeting SFKs with afatinib led to combined growth suppression in *in vitro* and *in vivo* in cells with T790M. Globally, our results suggest that an unbiased mass spectrometry approach can identify codrivers of resistance that can be cotargeted to enhance efficacy of targeted agents.

Materials and Methods

Reagents

Gefitinib, erlotinib, afatinib, and WZ4002 were purchased from Chemie Tek (Indianapolis, IN). CL-387,785 was purchased from AXXORA (San Diego, CA).

Cell culture

The human H1975, H460, A549, and H1299 NSCLC cell lines were obtained from American Type Culture Collection. The human HCC4006 NSCLC cells were kindly provided by Dr. Paul Bunn (University of Colorado, Aurora, CO). The human HCC827 NSCLC cells were provided by Dr. Jon Kurie (MD Anderson Cancer Center, Houston, TX). The human PC9 NSCLC cell line was kindly provided by Dr. Hayata, Tokyo Medical University (Tokyo, Japan). PC9GR cells were generated by exposure of PC9 cells containing a TKI-sensitive EGFR mutation (exon 19; E746-A750) to gradually increasing concentrations of gefitinib, beginning at 3 nM and up to 2 μM, for 3 months. HCC4006-T790M and HCC827-T790M cells were generated as previously described (26). All cell lines have been maintained in a central repository at Moffitt since 2008. All cell lines had been authenticated by STR analysis (ACTG Inc, Wheeling, IL) as of September 2010, and all cells had been routinely tested and were negative for mycoplasma (PlasmoTest, InvivoGen, San Diego, CA). Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). Apoptosis assays were performed using PE-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Biosciences). Rescue experiments were done as previously described (27).

Genotyping

Total genomic DNA from parental and resistant cells was prepared using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) in accordance with the product manual. Direct DNA sequencing was used to detect EGFR mutations as previously described (28). We also applied the PCR-invader assay to detect minor populations of EGFR mutation, as previously described (29). MET gene copy number per cell was determined by fluorescence in situ hybridization with the use of the LSI D7S522 (7q31) Spectrum Orange and chromosome 7 centromere (CEP7) Spectrum Green probes (Vysis; Abbott), as previously described (30).

Tyrosine phosphoproteomics

Tyrosine phosphopeptides were purified according to the manufacturer's recommendations for the Cell Signaling PhosphoScan kit (P-Tyr-100) (Cell Signaling Technology). Briefly, 2 × 108 cells were lysed in urea buffer; extracted

Published OnlineFirst June 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1559

proteins (40–80 mg) were then reduced by dithiothreitol, alkylated by iodoacetamide, and then digested by trypsin. Peptide mixture was isolated from lysate using Sep-Pak C18 columns and then lyophilizated. Phosphorylated peptides were immunoaffinity purified using phosphotyrosine antibody after lyophilizated peptide mixture was dissolved. Volumes of phosphotyrosine peptides were then downsized to 20 μL by vacuum drying for further experiments. The further peptide mixture separation and phosphosite assigning have been previously described (31). To quantify each tyrosine-contained peptide, we calculated peak area [also called extraction ion chromatography (EIC)] using Label-free strategy and xCalibur as the tools. Identification and quantification of some obscure peptides were manually verified. After quantification, 774 phosphorylated tyrosine sites were identified. An in-house algorithm was implemented to identify unique phosphorylation tyrosine (pY) sites, remove redundant sites, and merge miss-cleaved peptides by using protein ID, peptide sequence, and phosphorylation start-site index, with quantification of peak areas. When only identifiable to the level of pairs of pYs (e.g., next to each other or up to ~11 amino acids apart), then the independent unit for analysis was the unique pY pair (instead of single site). Mis-cleaved phosphopeptides or fragments of the same phosphopeptides were merged. Peptides shared by multiple proteins were annotated. Among which, two pairs of sequences were potential results of co-elution and therefore not included in further analyses. A total of 524 unique phosphotyrosine units (pYs) or pY pairs were identified. Quantification and stability of 5 MYG peptides across samples were examined, with the average of 3 of them used for normalizing the peak ratio areas across 16 samples (8 biological samples with technical duplicates) so that the normalized quantities across samples were comparable. Reproducibility between technical replicates for each pY was estimated using Pearson correlation. The corresponding *P* values were used to estimate false discovery rate. High technical reproducibility of FDR ≤1% was used in our study. In addition, if the pY was detected in at least half of the samples in this study, i.e., at least 8 of 16 samples, it passed the QC criteria. Among the 524 unique pY units, 403 of them passed the QC criteria and were included in the analyses. 377 of them were unique pY sites while 26 of them were unique pY pairs. Averages of technical replicates from 8 biological samples were used in the analyses. We used a simple imputation (i.e., when one of technical replicates is missing, the detected value from the other remaining technical replicate was used). Data were analyzed in log2 scale prior to parametric analyses and also for ease of interpretation. For example, the difference of 1 in log2 scale is a 2-fold change between two conditions. Two-way ANOVA with the interaction term was performed to answer the following three research questions: 1) Which tyrosine sites are differentially phosphorylated between the cell lines with and without drug resistance? 2) Which tyrosine sites are differentially phosphorylated between the control and erlotinib-treated groups? 3) Which pYs phosphorylation response to treatment is different between the resistant and non-resistant cell line? To adjust for multiple hypothesis testing, the resulting *P* values for the main effects of cell line and treatment as well as the cell line-by-treatment interaction term were used to estimate false discovery rate. We used FDR ≤20% to declare statistical significance. We further performed network analysis based on these potential candidates. Interactions among all identified tyrosine phosphorylated proteins were retrieved from the Molecular Interaction database (MINT) (32); the IntAct database (33); the Database of Interacting Proteins (DIP) (34); the General Repository for Interaction Datasets (BioGRID) (35) and the Biomolecular Interaction Network Database (BIND) (36) using InnateDB (37) and visualized in Cytoscape 2.8.3 (38).

Protein expression analysis

Western blot analysis of whole cell lysates was performed as described previously (27). Primary antibodies to EGFR, MET, pTyr 1234/1235 MET, IRS2, pTyr 1131 IGF1R, AXL, pTyr 702 AXL, Src, pTyr 416 Src, Akt, pSer 473 Akt, Erk, pThr202/Tyr204 Erk, and PARP were obtained from Cell Signaling Technology. Primary antibodies to pY1068-EGFR were obtained from Invitrogen (Carlsbad, CA). Primary antibodies to β-actin were purchased from Sigma-Aldrich (St. Louis, MO).

Assessment of tumor growth inhibition *in vivo*

All animal procedures were approved by our Institutional Animal Care and Use Committee. PC9GR cells (2×106) were injected subcutaneously into the flank of 7-week-old female athymic nude mice. The mice were divided into 4 treatment groups of 7 animals: those treated over 3 weeks by daily oral gavage of vehicle, afatinib (10 mg/kg), dasatinib (15 mg/kg), or both afatinib and dasatinib; 0.5% (wt/vol) aqueous solution of hydroxypropylmethylcellulose was used as vehicle for afatinib, and 50% propylene glycol was used as vehicle for dasatinib. Treatment was initiated when tumors in each group achieved an average volume of 100 mm^3, with tumor volume being determined twice weekly for 21 days after the onset of treatment from caliper measurement of tumor length (L) and width (W) according to the formula LW2/2.

Src-Tyr416 immunohistochemistry staining

Immunohistochemistry staining was performed to measure the expression of phosphor-Src (Tyr416) in paraffin tissues from 10 lung cancer patients with mutant-positive EGFR T790M.

Slides were stained for phosphor-Src (Tyr416) (mouse monoclonal antibody; Millipore) using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) following the manufacturer's protocol with proprietary reagents. Briefly, slides were deparaffinized on the automated system with EZ Prep solution (Ventana). Enzymatic retrieval method was used in protease 1 at 4 minute (Ventana), CC1 Standard and CC2 standard conditions. The primary monoclonal antibody (Millipore) reacts to secondary antibody at different dilution-titrations. Both primary and secondary